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13. ABSTRACT (Maximum 200 words) We have initiated studies of programmed cell death, or apoptosis, in human breast cancer cells. Using the MCF-7 breast cancer cell line, we have found that cell growth is stimulated by supplementing the culture medium with 17β-estradiol, and inhibited by charcoal stripping serum to remove steroid hormones. We have found that MCF-7 cells are resistant to induction of apoptosis by the calcium pump inhibitor, thapsigargin, ostensibly due to expression of Bcl-2. We have found that estrogen withdrawal decreases the level of Bcl-2 expression, but does not increase the susceptibility of cells to apoptosis due to constitutive Bcl-2 expression.				
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FOREWORD

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INTRODUCTION

The overall purpose of the proposal is to investigate the role of calcium in the signal transduction cascade that mediates programmed cell death, or apoptosis, in breast cancer cells. The emphasis of this proposal is on basic mechanisms. It is anticipated that an improved understanding of the mechanism of apoptosis and how it is controlled in breast epithelium should facilitate the development of novel therapeutic strategies based on inducing apoptosis.

Apoptosis is a naturally-occurring form of cell death important for the proper development and homeostasis of many tissues (1,2). Apoptosis can be induced in breast cancer cells by estrogen ablation (3,4,5) or treatment with the antiestrogen tamoxifen, the antiprogesterin RU486 (3), or the somatostatin analogue, SMS 201-995 (6). Understanding the mechanism of apoptosis and how it is regulated will provide an opportunity for new therapeutic initiatives, as well as insight into the pathogenesis of breast cancer. In the latter regard, there is growing evidence that aberrant cell survival resulting from inhibition of apoptosis can lead to cancer (see ref.7 for review). Apoptosis is physiologically regulated by genes, such as Bcl-2 (2,8). Bcl-2 is expressed in many tissues that are characterized by apoptotic cell turnover, including breast epithelium (9). It seems highly likely that aberrant expression of Bcl-2 may interfere with apoptosis in breast cancer cells, a concept that is being tested in our studies.

One hypothesis which this proposal addresses is that mobilization of calcium from the endoplasmic reticulum (ER) may be a critical step in the apoptotic pathway of breast cancer cells, induced by a variety of events, including hormone withdrawal, antiestrogen treatment, and growth factor withdrawal. We also predict that thapsigargin (TG), a potent and selective ER calcium pump inhibitor will induce apoptosis in breast cancer cells, bypassing more proximal steps in the apoptotic pathway, such as hormone-receptor interaction. If this prediction is correct, then it might be possible to develop new therapeutic agents for use in treating hormone-resistant breast cancer, based on the model of TG-induced cell death. Moreover, we suspect that apoptosis in breast cancer cells will be regulated by Bcl-2, or newly recognized members of the Bcl-2 family, a concept that will be tested in the current proposal.

BODY

During the current year of funding, which represents year 1 of a 4 year grant award, our first priority has been to develop an optimal in vitro model system for investigating programmed cell death, or apoptosis, in breast cancer. We systematically surveyed the current literature, and also sought the advice of Dr. Gloria Heppner, Michigan Cancer Foundation. The overall conclusion from this research was that the MCF-7 cell line is the best characterized and most often used cell line. We therefore obtained this line from two different sources, ATCC and investigators in Dr. Stanton Gerson's laboratory at CWRU. The cell line stocks from ATCC grew poorly, for reasons that are not clear. Therefore, all of the work reported in this progress report was done with the other MCF-7 line stock.

In the first series of experiments, we characterized MCF-7 cell growth under various culture conditions. The purpose of these experiments was twofold. First, we

wished to become familiar with the morphological and growth characteristics of the cell line. Second, we wished to determine the effect of steroid hormones on cell growth and viability, thereby testing the hypothesis that withdrawal of estrogen from an estrogen-dependent breast cancer cell line would induce apoptosis. Our findings are summarized in Figures 1 and 2. The results indicate that MCF-7 growth is optimal in medium supplemented with serum that has not been charcoal extracted to remove estrogens. When cells were cultured in medium supplemented with charcoal-extracted serum, cell growth was retarded, but cell viability was maintained. The results in Figure 2 indicate that supplementation of culture medium with 17- β -estradiol induces cell proliferation in a dose-dependent manner, with maximal cell growth achieved at a concentration of 1 nM. Thus, the cell line employed in our studies is clearly estrogen responsive.

To determine whether or not hormone withdrawal induces apoptosis in MCF-7 cells, we cultured cells in the presence of charcoal-extracted serum for up to 8 days. Cells were stained with ethidium bromide and acridine orange and then examined for apoptotic morphology by fluorescence microscopy (10). No morphological evidence of apoptosis was observed. Furthermore, we analyzed cellular DNA derived from cells at various time points, up to 8 days during culture in charcoal-extracted serum. There was no evidence of DNA fragmentation, consistent with the conclusion that the cells did not undergo apoptosis upon estrogen withdrawal. Hence, although cell growth was hormonally regulated, there was no evidence that the cells underwent apoptosis upon hormone withdrawal.

In a second series of experiments, we wished to determine whether the endoplasmic reticulum (ER) calcium pump inhibitor, thapsigargin (TG), induces apoptosis in MCF-7 cells. To this end, MCF-7 cells growing under usual culture conditions (i.e., in serum that was not charcoal extracted) were treated with 50-150 nM TG for three days. Cell morphology was assessed by fluorescence microscopy, revealing no increase in apoptosis above background. These experiments were repeated using cells that were cultured in charcoal-extracted serum for up to 6 days. Once again, there was no increase in the proportion of apoptotic cells above that present in the absence of TG. From these experiments, we conclude that the stock of MCF-7 cells employed in these experiments is resistant to induction of apoptosis by the potent and selective ER calcium pump inhibitor, TG. These findings are significant, as we have shown that other types of cells (e.g., mouse and human T cells) are quite sensitive to apoptosis induction by TG (11,12).

In earlier work in this laboratory, we discovered that induction of apoptosis by TG in lymphoid cells is inhibited by overexpression of the anti-apoptotic oncogene, Bcl-2 (12). Therefore, in view of the preceding findings, we have measured the level of Bcl-2 in MCF-7 cells by Western blotting. The purpose of these experiments was primarily to determine whether or not MCF-7 cells are resistant to apoptosis due to expression of Bcl-2. Our findings (Figure 3) indicate that MCF-7 cells express Bcl-2. In view of emerging evidence that apoptosis in breast cancer cells may be hormonally regulated, we initiated a series of experiments to determine whether Bcl-2 expression might itself be hormonally regulated. We therefore cultured cells in phenol red-free medium supplemented with charcoal-extracted serum (to remove endogenous

estrogen), and measured the level of Bcl-2 over a period of 8 days. The level of Bcl-2 clearly decreased when cells were cultured under estrogen-free conditions (Figure 3). These findings suggested that the level of Bcl-2 expression in MCF-7 cells is regulated by estrogen.

In the preceding experiments, we have observed that one of our MCF-7 stocks constitutively expresses a significant level of Bcl-2 which is not completely downregulated after hormone withdrawal. MCF-7 cells express a mutant estrogen receptor that is suspected of being constitutively active (13). The relative level of mutant receptor compared to wild type receptor varies among different stocks of the MCF-7 line (14). Therefore, we hypothesize that constitutive Bcl-2 expression observed in our experiments is secondary to expression of a constitutively active mutant estrogen receptor that induces Bcl-2 expression in a hormone-independent fashion. To test this hypothesis, we have initiated several lines of experimentation. First, we have purchased monoclonal anti-estrogen receptor antibodies and will be using these to characterize wild type and mutant estrogen receptor expression in MCF-7 cell lines employed in our work. This is feasible because the well characterized, constitutively active mutant estrogen receptor is known to migrate with a reduced molecular weight compared to wild type receptor. Second, we have obtained from two sources (ATCC and Dr. Marc Lippman) the MB-MDA-468 breast cancer cell line. This cell line reportedly does not express Bcl-2 (15), and also does not express estrogen receptor (5). This cell line has been used by others to study apoptosis (5). We now have this cell line in culture and are initiating a detailed characterization of the line with regard to Bcl-2 and estrogen receptor expression, and susceptibility to apoptosis. We intend to express wild type, functional estrogen receptor in this cell line to determine if estrogen receptor expression induces Bcl-2 expression, or if estrogen receptor expression confers estrogen inducibility of Bcl-2 expression. To this end, we have developed collaborations with two investigators, Dr. Benita Katzenellenbogen and Dr. Donald McDonnell, who have generously provided us with estrogen receptor expression vectors for use in our studies. If these experiments are successful, our next step will be to prepare an expression vector encoding a mutant estrogen receptor from which most of the hormone binding region has been deleted. We will use this expression vector to express mutant, constitutively active estrogen receptor in MB-MDA-468 cells, thereby definitively testing the hypothesis that a constitutively active, mutant estrogen receptor confers constitutive elevation of Bcl-2 and inhibits apoptosis.

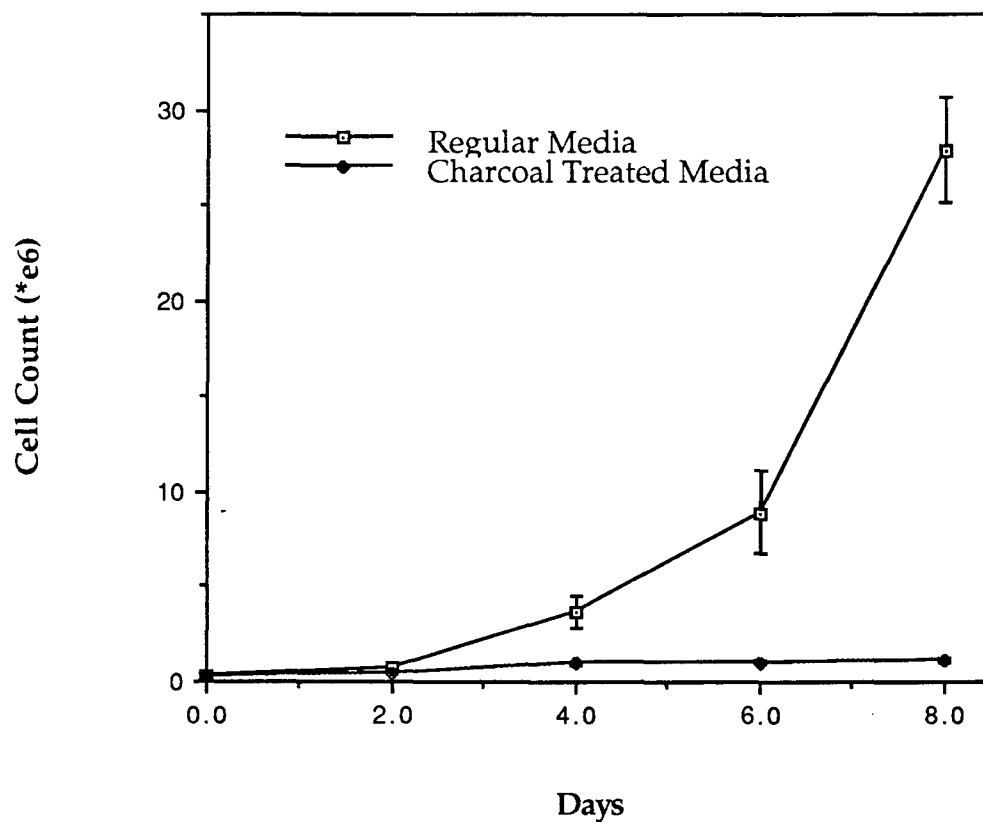
CONCLUSIONS

We have reached the following conclusions through the first year of work summarized above: (i) MCF-7 cells are resistant to apoptosis induction by the ER calcium pump inhibitor, TG, apparently due to expression of Bcl-2. (ii) In hormone responsive stocks of the MCF-7 line, Bcl-2 expression is induced by estrogen, and Bcl-2 expression decreases following estrogen withdrawal. (iii) Although Bcl-2 expression decreases following estrogen withdrawal, a constitutive level of Bcl-2 expression remains sufficient to inhibit TG-induced apoptosis, probably due to a mutant estrogen receptor that induces significant Bcl-2 expression in the absence of hormone.

Overall, the long term goals and specific aims of our research have not changed

during the first year of funding. However, in the second year of funding, the proposed studies will be expanded to include an analysis of the role of estrogen receptor mutations in inducing Bcl-2 expression.

Figure 1
Effect of serum charcoal extraction on MCF-7 cell growth



Effect of serum charcoal extraction on MCF-7 cell viability

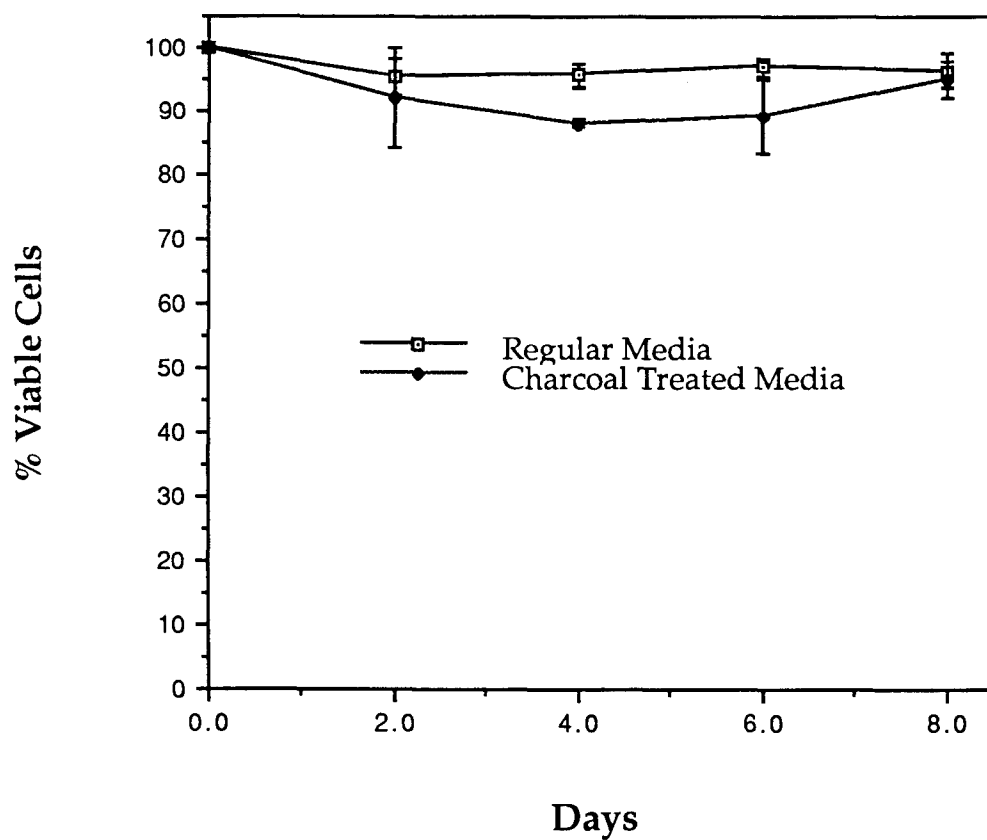
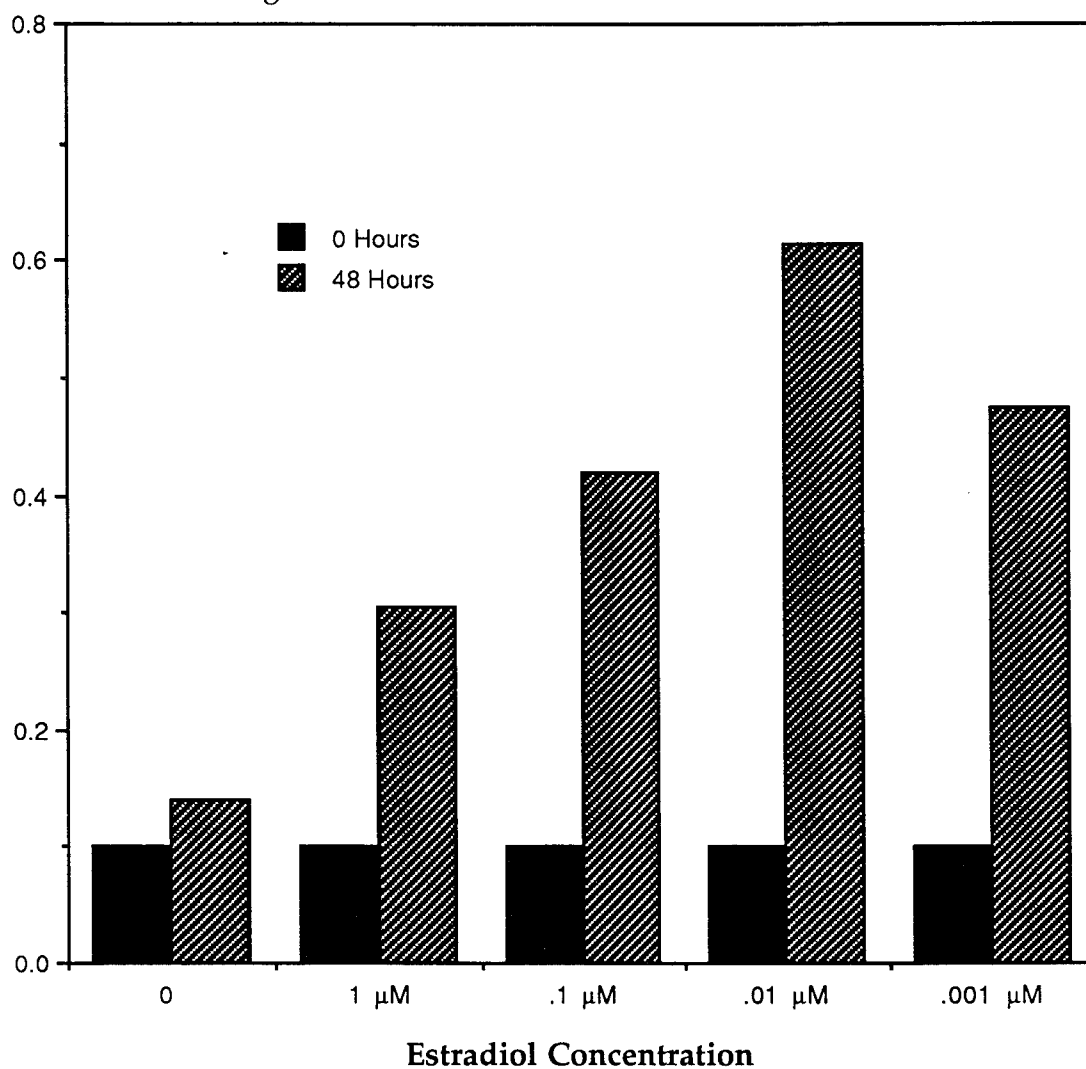


Figure 2

Effect of Estradiol on MCF-7 Cell Growth

MCF-7 cells were cultured for 0 and 48 hr with or without 17β -estradiol before counting the number of viable cells.



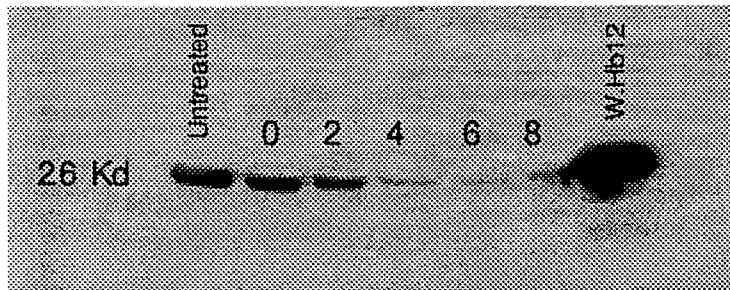


Figure 3

MCF-7 cells (untreated) were transferred to medium supplemented with charcoal-stripped serum and cultured for 8 days. At 48 hr intervals, protein was extracted and subjected to Western blotting using a monoclonal antibody to Bcl-2. A cell extract from WEHI7.2 mouse lymphoma cells that were stably transfected with a cDNA encoding Bcl-2 was used as a positive control.

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COLLEGES AND UNIVERSITIES RATE AGREEMENT

EIN #: 1341018992A1

DATE: February 13, 1995

INSTITUTION:
Case Western Reserve University
10900 Euclid Avenue
Cleveland

OH 48106-7006

FILING REF.: The preceding
Agreement was dated
March 17, 1994

The rates approved in this agreement are for use on grants, contracts and other agreements with the Federal Government, subject to the conditions in Section II.

SECTION I: INDIRECT COST RATES*

RATE TYPES: FIXED FINAL PROV. (PROVISIONAL) PRED. (PREDETERMINED)

TYPE	EFFECTIVE PERIOD		RATE(%)	LOCATIONS	APPLICABLE TO
	FROM	TO			
PRED.	07/01/91	06/30/96	51.0	On Campus	Research
PRED.	07/01/91	06/30/96	22.0	Off Campus	Research
PRED.	07/01/91	06/30/96	50.0	Univ Hospital	Research
PRED.	07/01/91	06/30/96	29.0	CMG Hospital	Research
PRED.	07/01/96	06/30/00	53.0	On Campus	Research
PRED.	07/01/96	06/30/00	26.0	Off Campus	Research
PRED.	07/01/96	06/30/00	51.0	Univ Hospital	Research
PRED.	07/01/96	06/30/00	42.0	CMG Hospital	Research
PROV.	07/01/00	UNTIL AMENDED	Use same rates and conditions as those cited for fiscal year ending June 30, 2000.		

*BASE:

Total direct costs excluding capital expenditures (buildings, individual items of equipment; alterations and renovations), that portion of each subaward in excess of \$ 25,000; hospitalization and other fees associated with patient care whether the services are obtained from an owned, related or third party hospital or other medical facility; rental/maintenance of off-site activities; student tuition remission and student support costs (e.g., student aid, stipends, dependency allowances, scholarships, fellowships).

TREATMENT OF FRINGE BENEFITS:

The fringe benefits are charged using a rate(s). Over/under recoveries from actual costs are adjusted in current or future periods. The directly claimed fringe benefits are listed in the Special Remarks section of this Agreement.

INSTITUTION:
Case Western Reserve University

AGREEMENT DATE: February 13, 1995

SECTION II: GENERAL

A. LIMITATIONS:

The rates in this Agreement are subject to any statutory or administrative limitations and apply to a given grant, contract or other agreement only to the extent that funds are available. Acceptance of the rates is subject to the following conditions: (1) Only costs incurred by the institution were included in its indirect cost pool as finally accepted: such costs are legal obligations of the institution and are allowable under the governing cost principles; (2) The same costs that have been treated as indirect costs are not claimed as direct costs; (3) Similar types of costs have been accorded consistent accounting treatment; and (4) The information provided by the institution which was used to establish the rates is not later found to be materially incomplete or inaccurate by the Federal Government. In such situations the rate(s) would be subject to renegotiation at the discretion of the Federal Government.

B. ACCOUNTING CHANGES:

This Agreement is based on the accounting system purported by the institution to be in effect during the Agreement period. Changes to the method of accounting for costs which affect the amount of reimbursement resulting from the use of this Agreement require prior approval of the authorized representative of the cognizant agency. Such changes include, but are not limited to, changes in the charging of a particular type of cost from indirect to direct. Failure to obtain approval may result in cost disallowances.

C. FIXED RATES:

If a fixed rate is in this Agreement, it is based on an estimate of the costs for the period covered by the rate. When the actual costs for this period are determined, an adjustment will be made to a rate of a future year(s) to compensate for the difference between the costs used to establish the fixed rate and actual costs.

D. USE BY OTHER FEDERAL AGENCIES:

The rates in this Agreement were approved in accordance with the authority in Office of Management and Budget Circular A-88, and should be applied to grants, contracts and other agreements covered by the Office of Management and Budget Circular A-21, subject to any limitations in A above. The institution may provide copies of the Agreement to other Federal Agencies to give them early notification of the Agreement.

E. OTHER:

If any Federal contract, grant or other agreement is reimbursing indirect costs by a means other than the approved rate(s) in this Agreement, the institution should (1) credit such costs to the affected programs, and (2) apply the approved rate(s) to the appropriate base to identify the proper amount of indirect costs allocable to these programs.

INSTITUTION:
Case Western Reserve University

AGREEMENT DATE: February 13, 1995

F. SPECIAL REMARKS:

FRINGE BENEFITS:

FICA	Benefits Salary
Retirement	Worker's Compensation
Life Insurance	Unemployment Insurance
Tuition Remission	Health Insurance
Severance Allowance	Dental Insurance
Disability Insurance	Consulting for Benefits

TREATMENT OF PAID ABSENCES:

Vacation, holiday, sick leave pay and other paid absences are included in salaries and wages and are claimed on grants, contracts and other agreements as part of the normal cost for salaries and wages. Separate claims for the costs of these paid absences are not made.

BY THE INSTITUTION:
Case Western Reserve University

(INSTITUTION)

(SIGNATURE)

R. James Henderson
(NAME)

Vice President for Finance & Admin
(TITLE)

March 2, 1995
(DATE)

BY THE COGNIZANT AGENCY
ON BEHALF OF THE FEDERAL GOVERNMENT:

DEPARTMENT OF HEALTH AND HUMAN SERVICES

(AGENCY)

(SIGNATURE)

John T. Glennon
(NAME)

ACTING DIRECTOR, DIVISION OF COST ALLOCATION
(TITLE)

February 13, 1995
(DATE) 5110

HHS REPRESENTATIVE: Henry Williams

Telephone: (214) 767-3261 x406

APPENDIX 2

Certificate of Environmental Compliance

CERTIFICATE OF ENVIRONMENTAL COMPLIANCE

The offeror currently ✓ IS IS NOT in compliance with applicable national, state, and local environmental laws and regulations. *(If not in compliance, attach details and evidence of approved mitigation measures.)*

The offeror has examined the activities encompassed within the proposed action entitled
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(enter title and/or Solicitation number and Principal Investigator's name), for compliance with environmental laws and regulations. The offeror states that the conduct of the proposed action WILL ✓ WILL NOT violate any applicable national, state, or local environmental law or regulation. *(If a violation will result, attach details describing the nature of the violation and evidence of approved mitigation measures.)*

The offeror agrees that if the work required under the proposed action at any time results in a violation of any applicable environmental law or regulation, the offeror will immediately take appropriate action, to include notifying the Contracting Officer, and coordinating with the appropriate regulatory agencies.

WARREN MALCHMAN, DIRECTOR
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AND ENVIRONMENTAL SAFETY
Title **10900 EUCLID AVENUE**
CLEVELAND, OHIO 44106-7227

Name of Organization


Signature

Date